INTRACELLULAR SYMBIOSIS IN COCKROACHES. I. PRODUCTION OF APOSYMBIOTIC COCKROACHES[1]

MARION A. BROOKS AND A. GLENN RICHARDS

Department of Entomology and Economic Zoology, University of Minnesota, St. Paul 1, Minnesota

Most of the literature on endosymbiosis is of a descriptive rather than an experimental nature, and the theories on the role of the symbiotes are usually based on circumstantial evidence. Practically all symbiote-bearing insects feed on diets which are incomplete or inadequate in certain substances known to be required by non-symbiotic insects or by vertebrates (Buchner, 1953). The only known symbiote-bearing insects among the general feeders are the ants, primitive termites, and cockroaches. Interestingly enough, parasites feeding on vertebrate blood do not possess symbiotes if during their larval stages they feed on a general diet, as is the case with mosquitoes, fleas and tabanids. It is also well known that among the Hemiptera-Homoptera, symbiotes are found only in the bugs which feed on vertebrate blood or plant juice but not in the bugs which are predacious on other insect species.

In any insect species which has intracellular symbiotes, the microorganisms have been found in every individual that was examined for them. Frequently the symbiotes are in anatomical relation to the insect's digestive tract. Thus it seems logical to ascribe to symbiotes a role in the nutrition of the insect host.

Hypotheses as to symbiotic functions are difficult to prove simply because in the majority of cases the two organisms are by nature inseparable. In those cases where the symbiotes are transmitted from one generation to the next as contaminants on the surface of the egg it is relatively easy to obtain aposymbiotic insects.[2] By surface-sterilization of eggs, larvae of a few insects have been obtained free of intestinal symbiotes and these larvae were unable to grow and reproduce normally (Koch, 1933; Schneider, 1940; Wigglesworth, 1952; Fraenkel, 1952). Pant and Fraenkel (1954) have actually identified certain B-vitamins and sterols provided by the yeast symbiotes of two beetles.

But when the symbiotes are intracellular and are transmitted in the cytoplasm of the egg to an intracellular location in the embryo, the sequence is far more difficult to interrupt. The only literature known to the present authors of removal

[1] Paper No. 3328, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul. The work described in this paper was supported by a contract between the Office of the Surgeon General, Department of the Army, and the University of Minnesota. The material presented was included in a thesis submitted by Marion A. Brooks to the graduate faculty of the University of Minnesota in partial fulfillment for the degree of Doctor of Philosophy.

[2] Several entomologists have queried our use of the word "aposymbiotic." It seems desirable to distinguish between insects which normally do not have symbiotes and those which normally do but which have been deprived of the symbiotes. The distinction is expressed by "asymbiotic" in the former case and "aposymbiotic" in the latter.

of intracellular symbiotes is the work of Aschner (1934) and Aschner and Ries (1933) on the body louse. In young embryos of this insect, the symbiotes congregate in a stomach disc, and during larval development the symbiotes migrate from the stomach disc to an ovarial mycetome from which they ultimately infect the eggs. If embryos were centrifuged so that the stomach disc, containing the symbiotes, was displaced, the symbiotes were unable to reach the mycetome. Larvae with uninfected mycetomes grew poorly and died prematurely. On the other hand, if infected mycetomes were surgically removed from normal larvae, the symbiotes were unable to reach the ovaries and this was followed by lack of egg development. These experiments indicate that the symbiotes are involved in both growth and reproduction.

The cockroach was one of the first insects recognized as having intracellular bodies presumed to be symbiotes (Blochmann, 1887). Blochmann discovered within the abdominal fat body discrete mycetocytes packed full of rod-shaped objects which he called *bacteroids*. Several later authors contributed information on the fine morphology of the bacteroids, the manner of their transmission, and the embryological development of the cockroaches (for reviews and complete bibliography, see Buchner, 1953; Steinhaus, 1947).

According to Buchner (1953), the presence of bacteroids has now been proved in 25 species of 16 genera of cockroaches so far examined. Although the microanatomy and details of transmission and embryological development vary from species to species, the general type of bacteroid-infection holds true throughout the whole order.

In brief, the bacteroids are always restricted to the mycetocytes (Fig. 1) of the fat body in both males and females except that in females some mycetocytes migrate to the ovaries in early nymphal life (Fig. 2). Bacteroids enter the ovarioles but the method of penetration of the tunica propria is unknown. The bacteroids remain in a peripheral layer within each oöcyte as it develops (Fig. 5).

In males, mycetocytes surround the testes (Fig. 6) of young nymphs but bacteroids have not been found in the testes and there is no evidence that bacteroids are transmitted by males.

During embryological development, the bacteroids are carried to the center of the egg with the cleavage nuclei. Although a variety of processes intervene at the next step in different species, in the subsequent development of the German cockroach, at least, the bacteroids get into the already-formed mycetocytes while the fat body is still segmented (Koch, 1949). Here again the method of migration of the bacteroids is unknown, as they give no evidence of motility. In young embryos of the German roach, the mycetocytes differentiate in clusters, one in each lateral half of abdominal segments 2 through 6. As development proceeds, the mycetocytes separate, increase by mitotic divisions (Brooks and Richards, 1955a), and become distributed throughout the visceral fat body of the abdomen. They do not enter the first, seventh, or eighth abdominal segments, the peripheral fat, or the thorax.

The visceral fat body of the abdomen is a diffuse, lobulated or branched tissue filling the hemocoel. It surrounds the intestinal canal and the gonads, and is itself enmeshed with tracheoles and Malpighian tubules.

The mycetocytes are distinctly different from the other cells of the fat body.

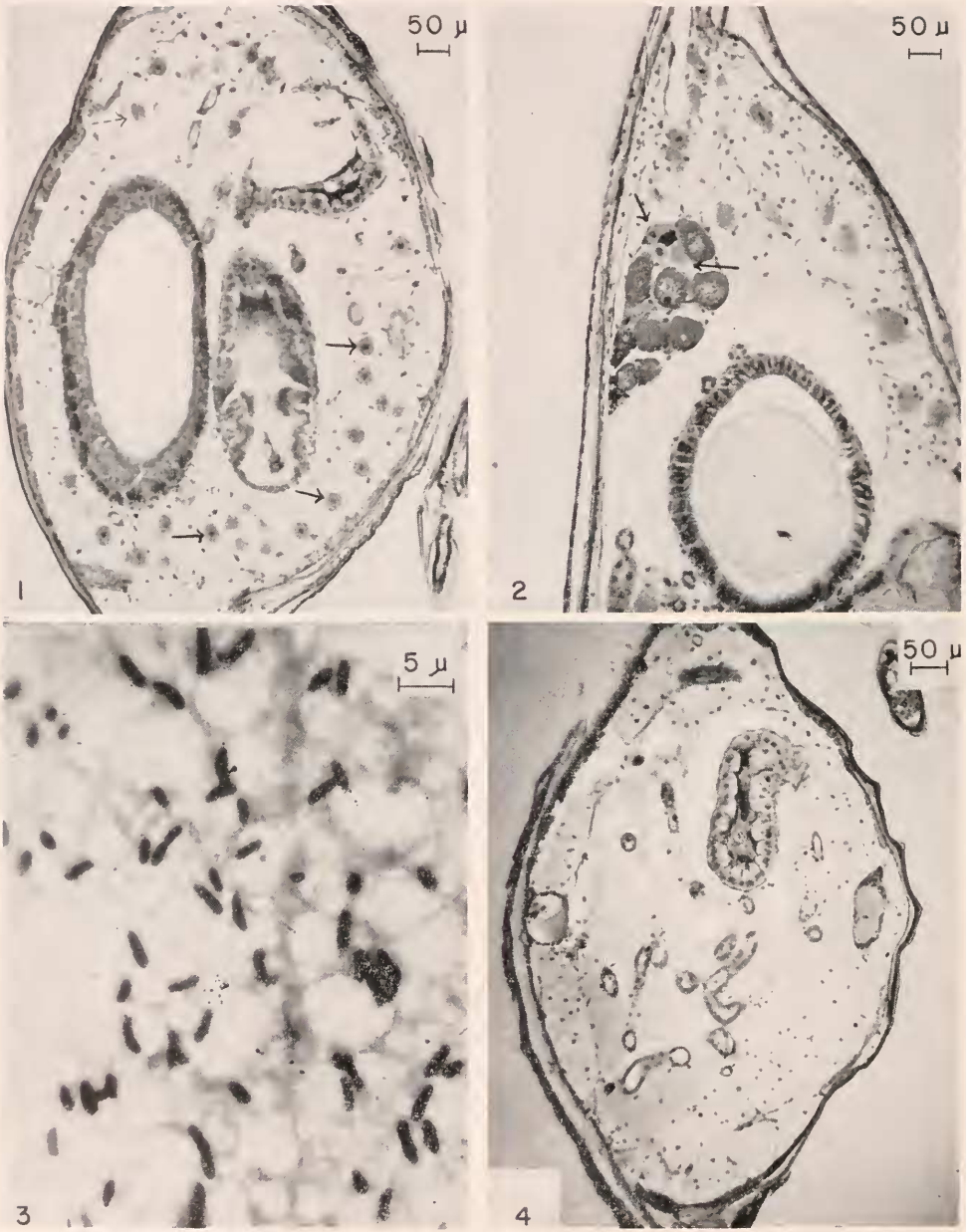

FIGURE 1. Mycetocytes (several indicated by arrows) in fat body as seen in cross-section of a three-week old German cockroach nymph. The section is turned so that the ventral side of the insect is on the right side of the photograph. Fixed in Carnoy's fluid, sectioned at 10 μ, stained with Delafield's hematoxylin and counterstained with clove oil saturated with erythrosin. After this treatment, the mycetocyte nuclei are blue and the bacteroids *en masse* are rose-violet or red. Individually the bacteroids appear hollow, the cell walls distinct and purple. The section was photographed with the aid of a Wratten red filter A (25).

The mycetocyte nuclei are relatively large and, in thin sections following Giemsa's stain, one may see that the cytoplasm appears fibrous. In normal roaches the mycetocytes are filled with bacteroids and have a sub-spherical or slightly stellate shape, with a diameter in the order of magnitude of 20 μ.

The method of transmission which has been described for cockroaches is called "hereditary transmission" or "transovarial infection."

Blochmann (1887) found that the bacteroids stained positively with Gram's stain. Figure 3 is a photomicrograph of Gram-stained bacteroids liberated from mycetocytes by crushing a piece of fat body on a microscope slide. The bacteroids in German roaches are approximately 3–6 μ in length and 0.9 μ in diameter. They are frequently seen in what appears to be a process of transverse constriction.

Numerous attempts to prove the bacterial nature of the bacteroids by culturing them have at best been ambiguous. Gier (1947) states that the most perplexing problem in symbiote cultivation is the identification of the cultured organism. This is true because there has not been a cockroach positively known to have been deprived of its microorganisms so that a modified statement of Koch's third and fourth postulates could be tested.

As Lederberg (1952) stated (p. 415) in respect to symbiotic problems in general, "Too little emphasis has been placed on the occurrence and behavior of 'disinfected' or aposymbiotic individuals, and on the criterion of re-infection for the specificity and identity of the endosymbiotic microorganism."

Brues and Dunn (1945) attempted to eliminate the bacteroids by injecting various sulfa drugs and penicillin into the large tropical roach, *Blaberus craniifer*. Doses of sulfa comparable to or higher than the mouse tolerance had no effect on the bacteroids. Penicillin, on the other hand, if given in tremendously large doses killed, or at least greatly reduced, the bacteroids (as observed in stained sections); but the cockroaches died. Since death did not result immediately, the authors thought it was caused by the lack of the bacteroids rather than by the toxic effects of the penicillin.

Glaser (1946) administered sodium sulfathiazole in the drinking water and injected sodium and calcium penicillin into the body cavities of adult American roaches, *Periplaneta americana*. He also subjected a few adults and some nymphs to prolonged high temperature (39° C.). About 38% of all his treated animals survived. Upon sacrificing the survivors, he observed that the bacteroids in the fat body and in the ovaries were either modified or absent and that the ovaries themselves had usually retrogressed. Glaser made the interesting observation that heat treatment of juveniles abnormally prolonged their development.

Noland (personal communication) confirmed Glaser's penicillin and heat effects and also extended sulfa treatments to include the German roach, *Blattella germanica*. In every instance where the bacteroids were reduced to the vanishing point, the ovaries were incapable of reproduction. Both Glaser and Noland based their diagnoses on Gram-stained smears.

FIGURE 2. Mycetocytes (indicated by arrows) within the ovary of a three-week old nymph. Prepared as above.

FIGURE 3. A smear of fat body from a German cockroach stained with Gram's stain. Bacteroids are Gram-positive.

FIGURE 4. Cross-section of a young aposymbiotic nymph. There are no mycetocytes or bacteroids in the section. Prepared as in Figure 1.

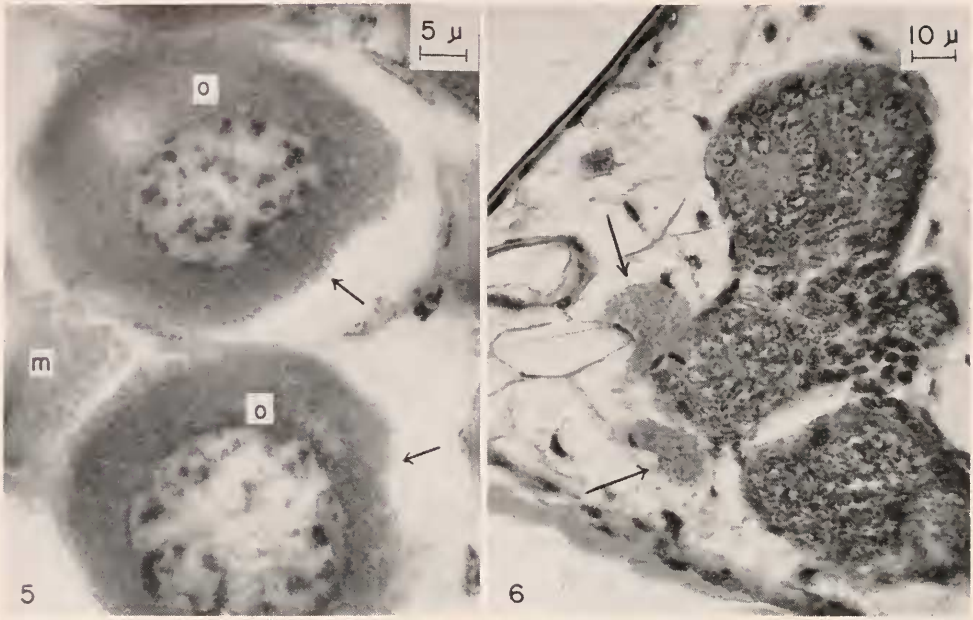

FIGURE 5. Bacteroids (appearing circular in section) forming a peripheral layer under the tunica propria of young oöcytes. m = mycetocyte; o = oöcyte. Prepared as in Figure 1.

FIGURE 6. Mycetocytes (indicated by arrows) appressed to or near the testes of a three-week old nymph. Prepared as in Figure 1.

Conclusive results were not obtained from the series of experiments mentioned above because it was impossible to distinguish the effects of the drugs or heat from the effects of the loss of the bacteroids.

The present study was planned as an extension and elaboration of the works of Brues, Dunn, Glaser, and Noland with the hope that among the newer antibiotics there would be one more effective in eliminating the bacteroids and yet allow the treated cockroaches to live and reproduce.

Materials and Methods

The German cockroach, *Blattella germanica* L., was used primarily because of its short life cycle. Another fact in its favor is its small size, which reduces the expense and labor of making serial sections of whole insects. The original colony was established by catching a few adults in the laboratory building. Females with ripe egg capsules were individually segregated in small cages and the nymphs, upon hatching, were randomly divided and distributed to several matched cages for testing antibiotic and dietary variables. In this way groups of 8 to 10 genetically similar insects were used for comparing sets of 4 differently-supplemented diets.

Records were kept of the dates of maturity, the appearance of the first egg capsules, the hatching of the first nymphs, and the death of the individuals of the first generation. The first filial generation was again divided and distributed and similar records kept for them, and in certain cases the second filial generation

was studied likewise. In this way pedigrees were established for three-generation spans on some of the diets.

A total of 18 different antibiotic-diet combinations was fed over a period of 15 months. Once the effects of the antibiotics or diets became known, the laborious record-keeping became unnecessary and larger pooled groups were used for obtaining growth curves. The nymphs were anesthetized with carbon dioxide gas, brushed clean of food and debris, and weighed in a vial on an analytical balance.

The crude diet used for feeding stock cultures of the cockroaches and for a base in some of the experimentally supplemented diets was a dog biscuit known as "Morton's Kibbies," obtained from the Morton Dog Food Company of Minneapolis. The content of this biscuit is given on the bag as follows:

corn meal
kelp
baking powder
second clear flour
brewers yeast
fish meal
soybean oil meal
feeding oat meal
linseed oil meal
Fleischmann's irradiated dry yeast
cod liver oil
meat and bone meal
standard wheat middlings
chlorophyllin (in a carrier of dehydrated alfalfa leaf meal)

The analysis of the biscuit is given as follows:

protein, minimum = 19.00%
fat, minimum = 2.50%
fibre, maximum = 4.00%
NaCl, maximum = 1.13%
Ca, minimum = 2.42%
P, minimum = 1.235%

The mineral content, less than 5%, is salt, potassium iodide, iron oxide, manganese sulphate, and calcium carbonate. After the ingredients are mixed, they are toasted at 380–390° F. (= 193–199° C.) for 32 minutes.

When this dog biscuit was used for experimental insects, it was ground in a food grinder and then finely pulverized in a ball mill.

The antibiotic and sulfa-drug supplements were from the following sources: chloromycetin (chloramphenicol), Parke, Davis and Company; aureomycin, crystalline, Lederle, Lot CP-103-1; aureomycin calcium drops, complimentary sample, Lederle Laboratories Division, American Cyanamid Company; sodium sulfathiazole (sesquihydrate) Merck U.S.P., complimentary sample, Merck and Company, Inc.; succinyl sulfathiazole Mann U.S.P., complimentary sample, Mann Fine Chemicals, Inc.

For heat treatments, the cockroaches were subjected to high temperatures of repeated short exposures in a heating chamber patterned after that of Noland (1944) or to somewhat lower temperatures for constant long exposures as Glaser (1946) had done.

Determination of the presence and condition of the bacteroids was made almost entirely by histological serial sections because smears proved unreliable. The material was routinely fixed in Carnoy's fluid, dehydrated through an ethanol-

butanol series, embedded in paraffin, sectioned at 10 μ, and stained with Delafield's hematoxylin followed by counter-staining with clove oil saturated with erythrosin. Flemming's fixing fluid and Heidenhain's iron hematoxylin stain were less satisfactory.

In some instances, fresh fat-body tissue or entire excised ovaries were incubated with neotetrazolium chloride by a special technique which stained only the bacteroids (Brooks and Richards, unpublished data).

Results

1. Effect of heat treatments

One brood of newly-hatched German cockroach nymphs was subjected on successive days to a 0.6° C. rise per minute until 40°, 41°, 42°, 43°, and 44° C. were reached on respective days. On the last day only 20 of the 30 insects recovered. Several were sacrificed for histological study and the rest were kept at 25° C. without further heating. Of the 14 survivors, only 5 lived to maturity. They appeared normal and produced offspring.

A second group of nymphs was brought to 40°, 42°, and 42° C. on three alternate days and maintained at those temperatures for 30 minutes. Several of these nymphs were sacrificed for histological sections immediately following the final heat treatment.

The sections from both treatments were practically identical. The heat had caused a gross emaciation of the fat body so that it was only a thin sheath instead of plump lobes. The fat cells were the component which had suffered, as they had lost most of their cytoplasm and were reduced to little more than nuclei and cell membranes. However, the only observable effect on the mycetocytes was that they were compactly rounded instead of stellate. As a net result the fat body consisted chiefly of mycetocytes. Some of the insects which survived the short heat treatments were sectioned 5 weeks later and at that time they presented a normal histological picture.

Constant high temperature of longer duration was then used in five other experiments. The insects were acclimatized to 37° C. at 50% relative humidity, which exceeded the incipient lethal temperature, *i.e.*, the highest temperature beyond which the insects could no longer live for an indefinite time. The effect of this temperature was studied on about 190 newly-hatched nymphs and 55 adults.

Exposing nymphs to 37° C. for 14 days caused the same shrinkage effect of the fat body as had resulted from higher temperatures of shorter duration. The subsequent growth and maturation of the nymphs was delayed slightly.

However, an exposure of between 17 and 22 days killed two-thirds of the nymphs by the time the heating period was ended and destroyed most of the bacteroids in the survivors. At the next molt, the cuticle of the survivors became a golden tan color instead of the normal dark brown-and-black. The subsequent growth of these nymphs was delayed by a period exceeding the length of the heat treatment; and only about one-third of them eventually reached maturity.

Smears made of fat-body biopsies of some of these retarded insects failed to indicate the presence of any bacteroids but subsequent complete sets of serial sections of the same individuals showed normal mycetocytes although their numbers were reduced. It is for reasons such as this that smears are unreliable indicators of the number of bacteroids.

The most adversely affected nymph was sacrificed 35 days after removal from the heat and although every section was examined, no normal mycetocytes were found. But a few bacteroids persisted in occasional mycetocytes.

One pair of cockroaches from this experiment produced offspring. About half of the eggs in the egg capsule did not develop, and of the formed embryos, only two hatched. The mycetocytes in one of these nymphs were not fully developed while those in the second were drastically retarded, containing only a few bacteroids.

Compared to nymphs, recently emerged adults were less resistant to 37° C. Two-thirds of the adults died in 10 days in contrast to 17–22 days for nymphs. The life span of the surviving adults was cut to less than one-third of the normal expectancy. The males were discarded as their sexual organs were badly damaged and normal males were mated to the treated females. Each female laid one abortive egg capsule before she died except for one individual which laid two capsules, nymphs hatching from the second capsule.

These experiments determined that 1) heat destroys some bacteroids; 2) heat treatment of nymphs delays subsequent growth; 3) heat treatment of adults retards reproduction; and 4) even though roaches which had been treated as nymphs seemingly recovered, certain adverse effects were passed on to the next generation. But definite conclusions as to the function of the bacteroids could not be drawn from these experiments because 1) it was impossible to distinguish the deleterious effects of the heat *per se* on both growth and reproduction from the lack of bacteroids; 2) it was impossible under the conditions of the experiments to *completely* eliminate the bacteroids without killing the cockroaches; and 3) the residual bacteroids in surviving insects evidently multiplied and approached a normal population.

2. Effects of feeding drugs and antibiotics

The crystalline drugs or antibiotics were ground with a mortar and pestle and thoroughly mixed with the pulverized dog biscuit. The cockroaches did not seem to object to the taste and consumed a normal amount of food. The levels of the doses were selected so as to be of the order of magnitude of the human daily dose with the difference that these doses were consumed throughout the insect's life. The levels were calculated on the basis of the known food consumption during the 300 ± days of a cockroach's life. A second level of doses was then mixed by arbitrarily adding 2 or 5 times the first amounts. The entire series of supplemented diets was as follows:

1) dog biscuit control
2) dog biscuit + 0.1% aureomycin
3) dog biscuit + 0.2% chloromycetin
4) dog biscuit + 1.0% sodium sulfathiazole
5) dog biscuit + 1.0% succinyl sulfathiazole
6) dog biscuit + 0.5% aureomycin
7) dog biscuit + 1.0% chloromycetin
8) dog biscuit + 2.0% sodium sulfathiazole
9) dog biscuit + 2.0% succinyl sulfathiazole

A second series made of a semi-synthetic diet with the same amounts of antibiotics was also fed, but the diet itself affected the bacteroids and those results will be reported in a separate paper (Brooks and Richards, 1955c).

The first time the diets were fed, divided litters were put on the diets as explained under Materials and Methods. Subsequently the experiment was repeated on a larger scale, one entire brood (usually 36 nymphs) being put on each diet and kept at a constant temperature of 27.5° C. The results of both experiments were comparable. When the roaches began to mature (at about 50–60 days), males and females from each diet were sectioned and stained with hematoxylin and erythrosin, and excised fat bodies from additional specimens from each diet were stained *in toto* with neotetrazolium chloride. The fat bodies of all roaches on the *low levels* of antibiotics appeared to have the normal number of mycetocytes, and the bacteroids were viable as judged by their ability to reduce tetrazolium. The fat bodies of the roaches on the *high levels* of aureomycin and sodium sulfathiazole had a reduced number of mycetocytes and the bacteroids in some of the remaining mycetocytes were no longer able to reduce tetrazolium. In fact, at the age of ninety days, other insects on these latter two diets were examined and no bacteroids could be found, either viable or otherwise. The ovaries of some of the females showed signs of deterioration. But all of the roaches on these diets were dead in six months (normal life span is about six months for males and one year for females). The fat bodies of the roaches on chloromycetin and succinyl sulfathiazole at either level were normal.

Aureomycin at both levels, sodium sulfathiazole at both levels, and succinyl sulfathiazole at the high level delayed the maturation of the cockroaches. These substances also caused a delay in the appearance of the first egg capsules, but more significantly, each female usually formed and aborted several egg capsules before one succeeded in hatching. The abortive egg capsules shriveled and dropped off after a few days, while German roaches normally carry their egg capsules for the period of incubation which is 21 to 28 days. No progeny were produced at all on the high level sodium sulfathiazole.

The growth of the roaches on the 2.0% sodium sulfathiazole was so slow that the first adults did not appear until two or two-and-a-half times the period required by the controls.

Although mortality on all of the high levels was considerable—25–50% after three or four months—the life spans on the low levels were not drastically shortened. The ages at death of one group (both sexes) on the dog biscuit control were between 224 and 396 days, while one group on 0.1% aureomycin lived for 187 to 348 days. The other experimentals lived to ages intermediate between those of the controls and the aureomycin-fed group. All of the early deaths on aureomycin were those of males. While the life span of normal males is between five and seven months, aureomycin-feeding shortened the life of males by as much as two months.

In short, administering antibiotics did not eliminate the bacteroids from the fat body of the cockroaches unless the dose was so high that it was accompanied by excessive mortality. However, the effect on the progeny of the treated roaches was quite another matter.

It was immediately obvious that there was something wrong with the offspring

of the aureomycin-reared parents. These nymphs were slightly smaller than normal, they were light gray in color instead of dark blackish-brown, and the embryonic cuticle, which is shed at the time of hatching, was not completely cast off but remained crumpled and attached to the anal cerci. The nymphs were weak and feeble. Some of them died immediately, and others lay on their backs for several days waving their antennae; but most of them were strong enough to withstand carbon dioxide anesthesia and careful handling. They were removed from the parental cages and fed pulverized dog biscuit. Most interesting was the nearly complete inability of these nymphs to grow on the stock diet. Control German nymphs on dog biscuit at room temperature molt every ten days, reaching the adult molt at approximately the age of sixty days. The individuals of this generation following aureomycin diet had not molted once by the end of thirty days, although exclusive of the deaths immediately following hatching, mortality was not much higher than among normal roaches and the nymphs ate and were lively.

Stained sections of representative samples of the nymphs revealed that they completely lacked bacteroids. In other words, we finally had aposymbiotic cockroaches! Usually no more than 24 nymphs hatched in each brood, whereas between 36 to 44 nymphs usually hatch from normal egg capsules. From every brood, 6 nymphs were taken at random and fixed for histological study while the remainder were used for growth studies. Complete sets of serial sections of the entire insects were carefully examined. No bacteroids were found. The fat body looked exactly like that of normal insects in areas in between mycetocytes except that in the aposymbiotic nymphs there were regions of anomalous tissue which later proved to be the "empty mycetocytes."

The appearance of the aposymbiotic fat body is shown in the photomicrograph, Figure 4, which may be compared with Figure 1, a photomicrograph of a cross-section of a normal nymph containing bacteroid-filled mycetocytes. Figure 7 shows one of the clusters of empty mycetocytes near an ovary of a young nymph. Figure 8 shows another cluster of empty mycetocytes in a more posterior abdominal segment.

Chloromycetin-produced offspring were normal in both histology and growth. A different type of response was elicited, however, by feeding either of the sulfa drugs. Offspring of parents on these diets were of three kinds: 1) normal in both histology and growth; 2) aposymbiotic; or 3) delayed in embryonic development. All three kinds of nymphs occurred in any one egg capsule. There were some bacteroids in the delayed nymphs, but the bacteroids were not enough to fill all of the mycetocytes. Consequently there were a few normally-filled mycetocytes, a few completely empty ones, and numerous partially-filled ones (Fig. 9). As a result of this inadequate complement of bacteroids, such delayed nymphs grew very poorly at first, but after a variable length of time (about a month), they began to grow and they matured at the age of approximately ninety days. If such delayed specimens were examined histologically after normal rate of growth had started, they looked normal. The mycetocytes apparently had become filled and distributed throughout the fat body.

The question logically arises: By what mechanism do the antibiotics break the chain in the hereditary transmission of the symbiotes to the next generation?

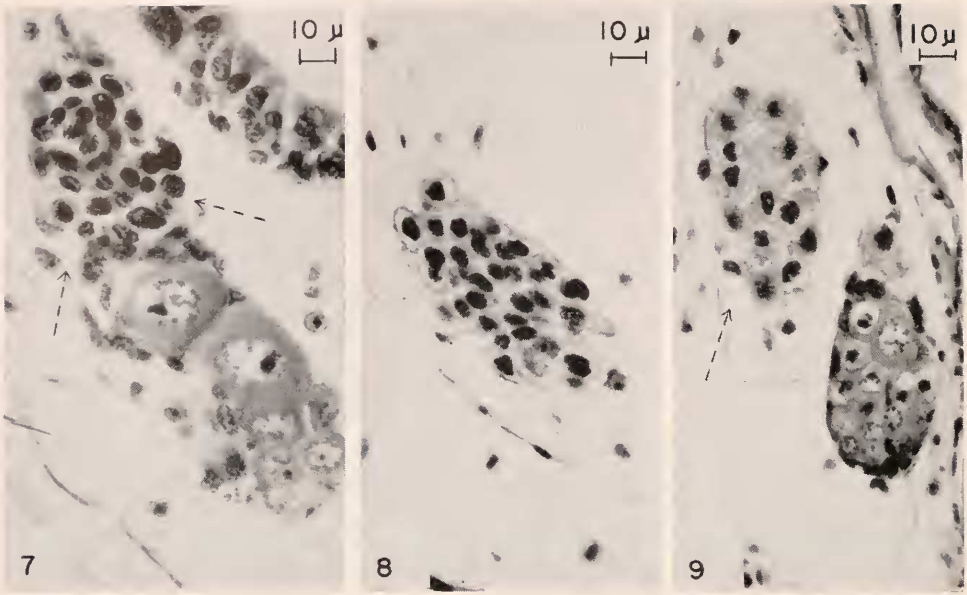

FIGURE 7. Part of a cross-section of a similar nymph as in Figure 4. A cluster of empty mycetocytes (indicated by arrows) is near the ovary. The nuclei of the mycetocytes are prominent but the cytoplasm is almost negligible in amount. The oöcytes are in the lower right side of the photograph. Prepared as in Figure 1.

FIGURE 8. Part of a cross-section of an aposymbiotic nymph showing a cluster of empty mycetocytes in the abdomen posterior to the ovaries. Prepared as in Figure 1.

FIGURE 9. Part of a cross-section through a 12-day old semi-aposymbiotic nymph, *i.e.*, one whose mycetocytes have an inadequate complement of bacteroids and are therefore delayed in development. The mycetocytes have not yet separated as they should normally have done during embryonic development. The ovary is on the right. Prepared as in Figure 1.

The break could be visualized by examining entire ovaries excised from young adult females reared on the various diets. The ovaries were stained with neotetrazolium chloride in such a manner that only the bacteroids were colored, all other tissue remaining colorless (Brooks and Richards, unpublished data).

Before elaborating on these results, it must be emphasized that the normal method of transmission is via the eggs to the embryo, and that the penetration of the ovaries by the bacteroids occurs at an early age. Figure 5 is a photomicrograph of a section of an ovary of a 3-week old nymph, in which some bacteroids have already left the mycetocytes and are now inside of the tunica propria. Such a preparation as this can be obtained equally well from roaches feeding on aureomycin. The antibiotic does not prevent the original infection of the ovaries.

But the tetrazolium-stained ovaries of mature insects show that the effect of the aureomycin takes place in the oldest egg of each series in the ovarioles. In an ovary of a normal female, the rose-violet color of the stained eggs is restricted to the periphery and is due to the reduced dye in the layer of bacteroids. The youngest oöcytes are stained intensely. The mature egg in each ovariole is lighter in color because the rapid enlargement of the ripening egg stretches thin the layer

of bacteroids. In an ovary of an aureomycin-reared female, some of the youngest ovules are colored, but the color lessens in the progressively older eggs until the oldest ones are completely white.

All of the examined females that had been reared on dog biscuit had at least *some* bacteroids in *some* of the immature ovules regardless of the antibiotic. On all diets that resulted in nymphs with bacteroids there were also stained bacteroids in the ripe eggs. Those cases which produced normal nymphs had ripe eggs deeply stained, while those cases which produced delayed mycetocytes had only a few bacteroids, probably less than 1% of the normal number, in the ripe eggs; so that grossly the eggs looked light pink or white instead of rose-violet. Those cases which produced nymphs without any bacteroids had very few bacteroids in the immature ovules and none in the ripe eggs. In most of these insects there were viable bacteroids in the fat-body mycetocytes. This leaves the oldest egg, which is rapidly growing, as the first site of complete destruction of the bacteroids.

The aureomycin effect is obtained only by feeding the antibiotic incessantly. This was determined by feeding one group (A) of cockroaches aureomycin (0.1%) until they matured, at which time they were transferred to control diet. Conversely, another group (B) was fed control diet until they matured, when they were transferred to aureomycin. In this way, the females of group A were eating normal diet while their eggs matured. The first nymphs of this group had either no bacteroids or at most only three or four per mycetocyte. During the first nine weeks following removal to normal diet, the successive hatches of nymphs had more and more bacteroids until finally the mycetocytes were normal. The first offspring of group B, which were eating aureomycin while the eggs matured, showed unmistakable signs of malformation of the mycetocytes; and the effect became more pronounced until by thirteen weeks the nymphs were completely aposymbiotic. Thus aureomycin began to affect the eggs within one week but the transmission was not completely blocked until after about three months.

The growth of the nymphs resulting from both groups A and B was directly proportional to the amount of normal mycetocytes in their fat bodies. That is, nymphs that grew well were found to have numerous mycetocytes; those which remained stationary lacked mycetocytes; while an intermediate series, which grew poorly, eventually possessed a few gigantic mycetocytes. The giant mycetocytes seemed to be the result of unchecked growth of the few mycetocytes which had each received only three or four bacteroids from the egg (Brooks and Richards, 1955a). The various subnormal mycetocytes in these nymphs also became attached to the ovaries.

As mentioned earlier, aureomycin shortens the life span of males; but it also, in some unknown way, affects the ability of the males to fertilize the eggs. When both sexes were reared on the antibiotic, there was a high percentage of inviable eggs. If aureomycin-reared females were mated to normal males, most of the eggs hatched. In fact, the analysis was carried one step further by mating normal females to aureomycin-reared males, which resulted in almost as many inviable eggs as from treatment of both sexes.

Seemingly the effect on the males is not one of behavior, as they were observed copulating. It is more likely a direct effect of the symbiotes on the sperm. In

normal males, mycetocytes are found close to or attached to the testes (Fig. 6), although bacteroids have never been seen in the testes and there is no evidence for transmission of bacteroids via the sperm. In aposymbiotic males, the empty mycetocytes also migrate to the testes as they do to the ovaries. The lack of bacteroids impairs the reproductive capacity of the aposymbiotic generation males to about the same extent that aureomycin does in the first generation. More will be said of this below.

3. Growth of aposymbiotic nymphs on crude natural diets

Reference has frequently been made to the fact that aposymbiotic nymphs, which themselves have not been fed drugs, are incapable of normal growth on the same crude diet, consisting of well-balanced natural foods, which supports growth of symbiotic cockroaches. An effort was made to replace the function of the bacteroids by feeding them to the nymphs. To this end, pieces of fat body (with bacteroids) freshly excised from normal nymphs were mixed with ground dog biscuit, a little sugar, and water to make a paste. This was renewed twice weekly. Similarly dried brewers yeast and alcohol-insoluble liver fraction (Nutritional Biochemicals Company) were also made into pastes and fed. The nymphs eating the yeast grew slowly and eventually all matured between the ages of 140–154 days, compared to 60 days for normal nymphs. Those eating the liver fraction grew even more slowly, the first one maturing only at the age of 189 days. The fat body did not enhance growth at all. The nymphs on this diet, as well as on the unsupplemented diet, were still immature at 266 days and weighed only between 4 and 7 milligrams. (The adult weights of German roaches are fifty milligrams for males and a hundred milligrams for females. There is no sex-correlated difference until the last instar.)

The cuticle of the slowly growing aposymbiotic nymphs was tan rather than the normal black and brown.

Although eating bacteroids did not improve growth, eating the excreta (accidentally or otherwise) of normal cockroaches did have a slight beneficial effect. Nymphs put in cages with normal nymphs, with normal adults, or even in cages without other insects but which had been soiled by them, all grew significantly better than nymphs isolated in clean cages. Moreover, the ones in the empty but soiled cages grew best of all. One individual has been kept as a curiosity with adults and is now over a year old and about half grown. Evidently the accessory growth factor is present only in the excreta and the improved growth in the presence of other insects is not a trophallaxis.

A few of the nymphs that were fed fat body or excreta were sectioned and stained. It was found that they had not become reinfected with bacteroids. (For the results of implanting tissue, see Brooks and Richards, 1955b).

Since feeding fat body was ineffective, mixing the diets as pastes was unnecessary and in the next trial the diets were fed as dry powders. The supplements were dried brewers yeast, dried alcohol-insoluble liver fraction, dried egg yolk (spray process, Fletcher-Eichman Company), and uncooked wheat germ (breakfast cereal, ground with mortar and pestle). A fifth diet was made of equal parts of each of the other four so that the final percentage of any one of the supplements was one-fourth as much as when it was the only supplement. Figure 10 shows

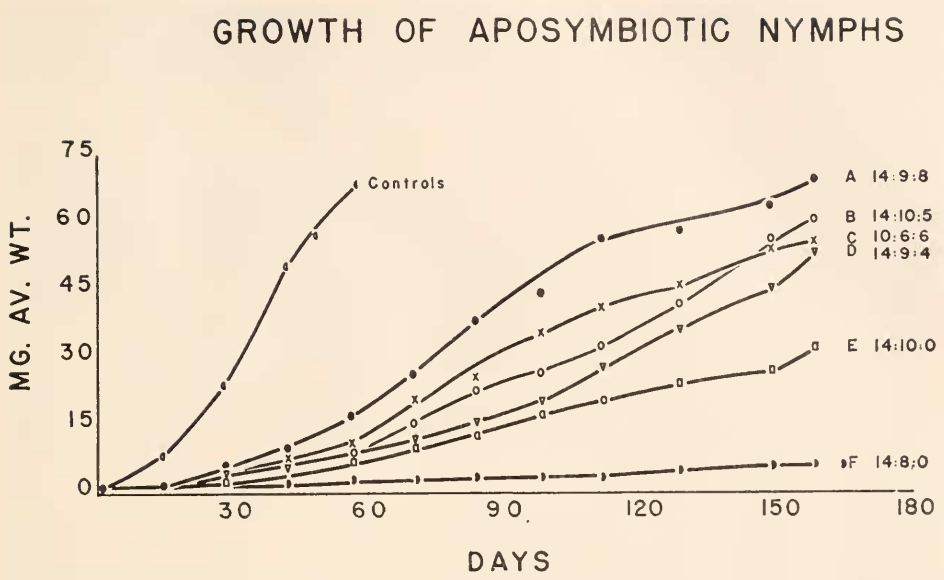

FIGURE 10. Growth of normal and aposymbiotic German cockroach nymphs. The insects were reared at approximately 25° C. The controls were normal nymphs fed unsupplemented dog biscuit. They had all reached maturity by the age of 57 days. The aposymbiotic nymphs were fed dog biscuit with supplements as follows (except F): A, 50% dried brewers yeast; B, 30% fresh wheat germ; C, equal parts A, B, D and E: D, 15% dried egg yolk; E, 10% dried alcohol-insoluble liver fraction; F, unsupplemented. The numerals after the key letters indicate the number of nymphs at the start of the experiment : the number alive when the experiment was terminated : the number which had matured.

the growth curves obtained. The numbers following each label represent the number of insects at start : number of insects at finish : number of insects maturing to adults. It can be seen that again a diet of 50% brewers yeast enabled the nymphs to mature in about two or three times the period required by normal nymphs. This seems to be an inordinately high amount of yeast, but in several preliminary trials, percentages of 25, 12.5, 10, and 5 were less effective in that order.

In order to dispel any doubts that it is the lack of bacteroids instead of a carrying-over of toxic effects of the antibiotics which prevents growth, we kept individual records on the performance of one litter from sulfa-fed parents. Six of the nymphs were killed immediately after hatching and they established the expected pattern of part symbiotic, part aposymbiotic nymphs. Twenty remaining nymphs were equally and randomly distributed to diets consisting of dog biscuit control, and yeast, liver, and fat-body supplements. It turned out that there was at least one insect with bacteroids on each diet, and each of these insects grew well and matured. The roaches without bacteroids grew poorly and failed to mature on the control and fat body-supplemented diets. The roaches without bacteroids grew slowly and eventually matured on the yeast- and liver-supplemented diets. These were not guesses: the insects were weighed individually and each one finally sectioned and stained. Since the nymphs were litter-mates, any toxic effect of

the drug fed to the mother should be expected to have affected them all alike. On the contrary, the lack of growth was directly correlated with the lack of bacteroids.

4. Reproduction of aposymbiotic roaches

Crosses were made between the three possible combinations of normal and aposymbiotic roaches after they had matured on the high yeast diet. The absence of the bacteroids did not result in early dissolution of the ovaries nor in complete suppression of the capacity for reproduction. However, there was a definite inhibition of reproduction, and this was true of males as well as of females. Continued feeding of yeast to the adults did not improve reproduction over that on an unsupplemented diet.

The method of testing these crosses was as follows: each aposymbiotic female was paired with a normal male; and each aposymbiotic male was mated to three recently emerged virgin females. A record was kept of the number of egg capsules produced in each cage.

Egg capsules produced by normal cockroaches almost invariably hatch if they are fertilized and if they are not damaged. However, 7 aposymbiotic females mated to normal males produced a total of 11 abortive egg capsules before nymphs hatched from 2 capsules. These and all subsequent offspring were without bacteroids. The 18 normal females mated to 6 aposymbiotic males produced 10 abortive egg capsules before nymphs hatched from 6 capsules. At the age of 20 days these nymphs molted to second instar, which is a normal rate of growth. When sectioned, they were seen to have normal mycetocytes.

Several litters hatching in the cages in which both parents were aposymbiotic constituted the second generation of cockroaches without bacteroids. Their histology and behavior were the same as those of the first generation. These have now been continued to the third aposymbiotic generation with the same properties being maintained.

Discussion

The endosymbiotic relationship of cockroaches and bacteroids is certainly of great antiquity. The recent discovery of a similar symbiosis in *Mastotermes*, a Carboniferous connecting link between the Isoptera and the Blattariae, places the relationship in the roaches as at least 300 million years old (Buchner, 1953; Koch, 1938).

There are several theories prevalent as to how endosymbiosis arose. Koch (1949) found that the mycetocytes form in the embryo of the German cockroach in anticipation of the reception of the bacteroids. The cells lie at the edges of the segmented fat body adjacent to the mid-gut and after their infection they sink deeply into the fat body. That the mycetocytes persist as specialized cells even in the absence of the bacteroids was established in the experiments reported in this paper. Possibly the mycetocytes originated phylogenetically as specialized cells within the intestinal epithelium, where they become infected with bacteroids contaminating the food. Their evolution would then have been toward removal from the epithelium and complete submersion in the fat body. This position would have necessitated the intervention of ovarian infection to insure transmission.

The restriction of the bacteroids to the mycetocytes may be the result of host immunization, as Glaser (1920) suggested, but the end result is the protection of the bacteroids. The relative imperviousness of the fat body to many influences such as extremes of osmotic pressure, temperature, food, and antibiotics undoubtedly protects the bacteroids within the mycetocytes. The bacteroids are peculiarly susceptible to destruction when they leave the mycetocytes. While in the developing ovum they can be killed by heat or antibiotics, and also in the developing ovum they either perish or fail to reproduce themselves if the host is consuming an incomplete diet (Brooks and Richards, 1955c). Furthermore, the bacteroids failed to establish themselves when they were injected as a suspension into the hemocoel (Brooks and Richards, 1955b).

Cockroaches deprived of bacteroids by breaking the chain of hereditary transmission by any of the methods mentioned above cannot live normally on a crude natural diet that is adequate for symbiotic roaches. There is a high percentage of mortality in the newly-hatched nymphs and growth is extremely slow in the survivors. While it is true that cockroaches are omnivorous, their food supply is meager at best and frequently unbalanced. If the diet of the aposymbiotic nymphs is fortified with highly nutritious foods, growth proceeds but at a slower rate than normal. Reproduction of adult aposymbiotic roaches, both males and females, is also deleteriously affected, most of the egg capsules, particularly the first several, being non-viable. The total result of depriving the insects of their symbiotes is thus one of *delay* in both growth and reproduction.

Since the bacteroids can be partially compensated for by a vitamin-rich diet, perhaps the function of the bacteroids is the production of a vitamin(s). But since the amount of vitamin-containing food that is required is out of all proportion to known vitamin requirements, it seems that the factor(s) needed is either 1) unknown and present in low concentration, or 2) not used *per se* but serves as a precursor of a second factor(s), such as a co-enzyme, the synthesis of which is aided by the bacteroids. The bacteroids themselves do not constitute a store of the required substance, because eating bacteroid-containing fat body did not result in re-infection of the aposymbiotic nymphs and did not permit normal growth.

Heat, aureomycin, and sulfa drugs were not equally effective in preventing transmission of the bacteroids to the next generation of roaches. The sulfa drugs were unreliable as they produced variable results. Aureomycin was completely effective and the results could always be duplicated. Heat treatments were not only less effective in preventing transmission, but the high temperature itself was fatal to a majority of the roaches, special equipment was needed for maintaining constant temperature and humidity, and there were difficulties in keeping drinking water available for the insects. In spite of these objections against using heat and sulfa, the results were worth the effort because they gave independent verification of the results from aureomycin. Regardless of which method was used, aposymbiotic offspring had the following characteristics in common: lack of growth on normal diet, slow growth with brewers yeast added to the diet, light colored cuticle, and poor reproduction.

First generation roaches suffering destruction of most of their bacteroids by either heat or a high level of sodium sulfathiazole stopped growing, but when yeast was added they resumed growth.

We have no suggestions to offer as to the processes by which the high temperature (37° C.) destroyed the bacteroids, but it seems appropriate to mention at this time that there are numerous reports in the literature on the inability to culture bacteroids at the standard incubator temperature of 37° C.

The specific effect of low level aureomycin on the bacteroids in the eggs as against those in the fat body is provocative. There may be a more rapid rate of metabolism in the enlarging eggs so that the antibiotic turn-over is stepped up, thus effectively increasing the dose acting on the bacteroids. This turn-over may be thought of as mitigated by either the egg protoplasm or by the bacteroids. On the other hand, instead of a quantitative difference between eggs and fat body, there might be a qualitative difference between the metabolism of the two tissues. This probably could occur if the chemical structure of the antibiotic resembles that of certain precursors needed for building egg protoplasm. And finally, there is the possibility that the bacteroids in the ripening eggs are simply prevented from reproducing themselves rather than killed outright.

There is no obvious reason why the various antibiotics should have acted as differently as they did. According to Merck's Index (1952), aureomycin is active against certain Gram-negative and Gram-positive bacteria, rickettsiae, protozoa, and viruses. Chloromycetin is active against all the preceding organisms except the protozoa, while sodium sulfathiazole is active against many bacteria. The effect of succinyl sulfathiazole is surprising inasmuch as this drug is commonly thought not to be absorbed and therefore effective only against enteric bacteria.

Theoretically, one may object to admitting that the evidence presented in this paper proves that the bacteroids are necessary for normal growth. The objection states that the bacteroids may be accumulated products of growth, and since the nymphs cannot grow as a result of the drugs given their parents, the bacteroids are not accumulated. However, stained sections of aposymbiotic roaches which *did* grow to maturity on yeast-diet contained no bacteroids. If the bacteroids are products of growth, they should have been accumulated in these insects.

The whole problem of the relationship of the bacteroids to the testes needs to be thoroughly investigated. The anatomical association of the mycetocytes with the testes and the impaired fecundity of both aureomycin-fed and aposymbiotic males all indicate that the bacteroids are of more significance to the male than has hitherto been suspected.

The function of the bacteroids as related to reproduction seems to be the supplying of a factor which, in the absence of the bacteroids, is not necessarily absent but available only in small amounts. The presence or absence of the bacteroids does not have an all-or-none effect on either reproduction or growth.

We wish to thank Dr. Jerre L. Noland for numerous valuable suggestions, and especially for pointing out the advantages of the German cockroach for the particular experiments needed.

Summary

1. The trans-ovarial inheritance of fat-body intracellular symbiotes in the German cockroach was prevented by subjecting the parent insects to high temperature or by feeding the parents aureomycin or sulfathiazole.

2. The most certain and simple method of obtaining aposymbiotic nymphs is by feeding the parents ground dog biscuit plus 0.1% aureomycin *all* of their lives.

3. Aposymbiotic nymphs are practically incapable of growth on a natural diet which is adequate for symbiotic nymphs.

4. The addition of large amounts of dried brewers yeast to the diet enabled the aposymbiotic nymphs to grow to maturity in two to three times the period required by normal nymphs.

5. Adult aposymbiotic cockroaches suffered impaired reproductive ability. The males were affected as well as the females.

6. The second and third generations of aposymbiotic roaches are similar to the first in both histology and behavior.

LITERATURE CITED

Aschner, M., 1934. Studies on the symbiosis of the body louse. 1. Elimination of the symbionts by centrifugalisation of the eggs. *Parasitol.*, **26**: 309–314.

Aschner, M., and E. Ries, 1933. Das Verhalten der Kleiderlaus bei Ausschaltung ihrer Symbionten. Eine experimentelle Symbiosestudie. *Zeitschr. Morph. Ökol. Tiere,* **26**: 529–590.

Blochmann, F., 1887. Ueber das regalmässige Vorkommen von bakterienähnlichen Gebilden in den Geweben und Eiern verschiedener Insecten. *Zeitschr. f. Biol.*, **24**: 1–16.

Brooks, M. A., and A. G. Richards, 1955a. Intracellular symbiosis in cockroaches. II. Mitotic division of mycetocytes. *Science* (in press).

Brooks, M. A., and A. G. Richards, 1955b. Intracellular symbiosis in cockroaches. III. Reinfection of aposymbiotic cockroaches with symbiotes. *J. Exp. Zool.* (in press).

Brooks, M. A., and A. G. Richards, 1955c. Intracellular symbiosis in cockroaches. IV. Effect of diet. *Ann. Ent. Soc. Amer.* (in press).

Brues, C. T., and R. C. Dunn, 1945. The effect of penicillin and certain sulfa drugs on the intracellular bacteroids of the cockroach. *Science,* **101**: 336–337.

Buchner, P., 1953. Endosymbiose der Tiere mit pflanzlichen Microorganismen. Verlag Birkhäuser, Basel. 771 pp.

Fraenkel, F., 1952. The role of symbionts as sources of vitamins and growth factors for their hosts. *Tijdschr. voor Ent.*, **95**: 183–196.

Gier, H. T., 1947. Intracellular bacteroids in the cockroach (*Periplaneta americana* Linn.). *J. Bact.*, **53**: 173–189.

Glaser, R. W., 1920. Biological studies on intracellular bacteria. No. 1. *Biol. Bull.*, **39**: 133–145.

Glaser, R. W., 1946. The intracellular bacteria of the cockroach in relation to symbiosis. *J. Parasitol.*, **32**: 483–489.

Koch, A., 1933. Symbionten und Vitamine. *Naturwiss.*, **21**: 543.

Koch, A., 1938. Symbiosestudien. III. Die intrazelluläre Bakteriensymbiose von *Mastotermes darwiniensis* Froggatt (Isoptera). *Ebenda,* **34**: 584–609.

Koch, A., 1949. Die Bakteriensymbiose der Küchenschaben. *Mikrokosmos,* **38**: 121–126.

Lederberg, J., 1952. Cell genetics and hereditary symbiosis. *Physiol. Rev.*, **32**: 403–430.

Merck and Co., Inc., 1952. The Merck Index. Rahway, N. J.

Noland, J. L., 1944. An improved method for the determination of the lethal temperature of insects, with special reference to studies on *Periplaneta americana. Proc. Indiana Acad. Sci.,* **53**: 186–187.

Pant, N. C., and G. Fraenkel, 1954. Studies on the symbiotic yeasts of two insect species, *Lasioderma serricorne* F. and *Stegobium paniceum* L. *Biol. Bull.,* **107**: 420–432.

Schneider, G., 1940. Beiträge zur Kenntnis der symbiontischen Einrichtungen der Heteropteren. *Zeitschr. Morph. Ökol. Tiere,* **36**: 595–644.

Steinhaus, E. A., 1947. Insect microbiology. Comstock Publishing Company, Ithaca, New York. 763 pp.

Wigglesworth, V. B., 1952. Symbiosis in blood-sucking insects. *Tijdschr. voor Ent.,* **95**: 63–69.